Linking environmental carcinogen exposure to *TP53* mutations in human tumours using the human *TP53* knock-in (Hupki) mouse model

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Abstract

TP53 is one of the most commonly mutated genes in human tumours. Variations in the types and frequencies of mutations at different tumour sites suggest that they may provide clues to the identity of the causative mutagenic agent. A useful model for studying human *TP53* mutagenesis is a partial human *TP53* knock-in (Hupki) mouse containing exons 4–9 of human *TP53* in place of the corresponding mouse exons. For an *in-vitro* assay, embryo fibroblasts from the Hupki mouse (HUFs) can be examined for the generation and selection of *TP53* mutations, as mouse cells can be immortalised by mutation of *Tp53* alone. Thus far four environmental carcinogens have been examined using the HUF immortalisation assay: (*i*) UV light, which is linked to human skin cancer; (*ii*) benzo[*a*]pyrene, which is associated with tobacco smoke-induced lung cancer; (*iii*) 3-nitrobenzanthrone, a suspected human lung carcinogen linked to diesel exposure; and (*iv*) aristolochic acid, which is linked to Balkan endemic nephropathy-associated urothelial cancer. In each case a unique *TP53* mutation pattern was generated that corresponded to the pattern found in human tumours where exposure to these agents has been documented. Therefore, the HUF immortalisation assay has sufficient specificity to make it applicable to other environmental mutagens that putatively play a role in cancer aetiology. Despite the utility of the current HUF immortalisation assay, it has several limitations that could be addressed by future developments, in order to improve its sensitivity and selectivity.

Abbreviations:

AA, aristolochic acid; AAN, aristolochic acid nephropathy; B[*a*]P, benzo[*a*]pyrene; BEN, Balkan endemic nephropathy; BPDE, benzo[*a*]pyrene-7,8-diol-9,10-epoxide; CYP, cytochrome P450; DBD, DNA-binding domain; HUF, Hupki embryo fibroblast; Hupki, Human *TP53* knock-in; LM-PCR, ligation-mediated PCR; MEF, mouse embryo fibroblast; 3-NBA, 3-nitrobenzanthrone; NER, nucleotide excision repair; PAH, polycyclic aromatic hydrocarbon; ROS, reactive oxygen species; WT, wild-type; UV, ultraviolet; Xpa, Xeroderma pigmentosum group A.

Introduction

Environmental factors including dietary habits and lifestyle choices play important roles in most human cancers, tempered by interindividual variation in susceptibility [1,2]. Cancer is a disease characterised by a series of genetic alterations that result in the loss of cellular growth, proliferation and differentiation control [3]. These genetic alterations include somatic mutations in DNA that may arise as a result of chemical action by agents of either endogenous (*e.g.* reactive oxygen species [ROS]) or exogenous (*e.g.* environmental carcinogens) origin. Initiation of carcinogenesis can occur through activating mutations in oncogenes (*e.g. RAS*), which encode proteins that promote cell proliferation and survival, and/or inactivating mutations in tumour suppressor genes (*e.g. TP53*), which encode proteins that normally suppress cell growth [4]. Initiated cells undergo clonal expansion as they are promoted by their microenvironment and accumulate additional mutations that endow the population with invasive, metastatic and angiogenic capabilities.

The most commonly mutated gene in cancer is the tumour suppressor *TP53*. Somatic mutations in *TP53* have been found in approximately 50% of human cancers [5], and rare *TP53* germline mutations (*e.g.* Li-Fraumeni syndrome) predispose carriers to various tumour types [6]. There is a large and diverse spectrum of *TP53* mutations that can lead to altered function of the gene product and contribute to malignant transformation. This diversity contrasts with other commonly mutated genes, such as *RAS*, where activating mutations occur in only a few codons of the gene [7]. Therefore, mutation spectra in *TP53* may be especially informative in attempting to understand the origin of mutations in human tumours.

TP53 encodes for the protein p53 that functions predominantly as a transcription factor, although other activities have been described [8]. Mice with genetic deletion of *Tp53* develop normally but are tumour prone, suggesting that p53 is not essential for normal cell growth but acts to prevent the growth of abnormal cells [9]. In normal, unstressed cells p53 protein expression is kept low via ubiquitin-mediated proteolysis that is regulated by the E3 ubiquitin ligase MDM2 [10]. However, p53 protein accumulates in response to various stresses, such as DNA damage, activation of oncogenes or hypoxia [11,12]. This occurs via post-translational modifications (*e.g.* phosphorylation and acetylation) that inhibit the interaction of p53 and MDM2 and can regulate its activity and location in the cell [13]. Once p53 is stabilised and activated it coordinates an appropriate response by activating the transcription of a variety of genes involved in cell cycle arrest, DNA repair, senescence and apoptosis [14,15]. For example, in response to genotoxic stress, p53 can transiently arrest the cell cycle at G1 or G2, such as by inducing the expression of $p21^{WAF1/Cip1}$, a cyclin-dependent kinase inhibitor [16]. This allows time for the cell to survey and

repair the damage, and prevents damaged cells from dividing. p53 can also induce senescence, which is a permanent G1 arrest. In cells that have been severely damaged, p53 may activate apoptosis by stimulating the transcription of genes such as *PUMA* and *NOXA* [17]. Disruption of the normal p53 response by *TP53* mutation contributes to transformation by eliminating the cell's braking mechanism in the face of stress and oncogenic activation.

TP53 mutations can be linked to cancer aetiology

Around 25,000 *TP53* mutations in human tumours have been registered in the International Agency for Research on Cancer (IARC) *TP53* database (www.p53.iarc.fr) providing an important resource for studying the types and frequencies of mutations in human tumours [18]. *TP53* contains 11 exons but most mutations are of the missense type in exons 5–8, which code for the DNA binding domain (DBD) of p53. Of the 1150 possible missense mutations in the DBD, 999 have been reported in tumours, as well as all 58 possible nonsense mutations [18]. Amidst the great variety of *TP53* mutations, several patterns have emerged [19]. The *TP53* mutations that are manifest in human tumours have been shaped by a combination of (*i*) the origin of the mutation (*e.g.* type of mutagen), (*ii*) the sequence of *TP53*, (*iii*) efficiency of lesion repair, and (*iv*) the selection for mutations that disrupt the normal function of p53. In principle, this information can be used to generate hypotheses regarding disease risk factors in a defined population [18].

Mutation patterns and spectra in *TP53* are often cancer specific [19], suggesting that environmental exposures may lead to a specific signature of mutations. Three often-cited observations that draw a link between a particular mutation profile and specific environmental risk factors are: (*a*) basal and squamous cell skin carcinomas caused by exposure to ultraviolet (UV) light that contain a high prevalence of tandem CC to TT transitions in *TP53* [20,21]; (*b*) lung tumours of tobacco smokers (but not of non-smokers) that contain a high percentage of G to T transversions in *TP53* at several hotspot locations, characteristic of polycyclic aromatic hydrocarbons (PAHs) present in tobacco smoke [22,23]; and (*c*) hepatocellular carcinoma from high incidence areas where aflatoxin exposure and chronic hepatitis B infection are common, that predominantly contain a G to T transversion at codon 249 of *TP53* [24,25]. More recently, a high prevalence of A to T transversions in *TP53* has been found in urothelial carcinoma associated with Balkan endemic nephropathy (BEN) and linked to exposure to aristolochic acid (AA) [26,27].

Base chemistry and sequence context play a key role in chemical- and UV-induced mutagenesis of *TP53*. One of the most important influences in the *TP53* sequence is the presence of CpG dinucleotides. The *TP53* DBD contains 23 CpG dinucleotides, all of which are methylated in human tissues [28]. Thirty-three percent of *TP53* DBD mutations and six major hotspots (codons

R175, R213, G245, R248, R273, and R282) occur at methylated CpG sites [29]. These sites are inherently promutagenic for two main reasons. Firstly, spontaneous deamination of 5-methylcytosine creates thymine and is thought to be a main source of C to T transitions in internal cancers [30]. Secondly, certain environmental carcinogens, such as PAHs, preferentially bind to guanines in methylated CpG sites, and UV irradiation often modifies methylated cytosines [31-33]. Thus, in cells exposed to such factors, mutations within methylated CpG sites may be most common.

The observed spectrum of *TP53* mutations has been further shaped by selection for mutants that exhibit loss-of-function and dominant-negative effects or, in some cases, gain-of-function. Approximately 80% of the *TP53* DBD missense mutations in tumours code for a protein with little or no transactivational capacity as shown using a yeast-based functional assay [34]. These mutants also commonly exert dominant-negative effects against wild-type (WT) p53 [18]. Mutations that have the greatest impact on p53 function will be selected for in tumourigenesis. For example, of the 34 possible missense mutations arising from transitions at CpG sites in the *TP53* DBD, only seven are frequently observed in tumours [35,36]. These are located in codons for amino acids which either bind directly to the DNA of target genes (R248, R273) or are critical for stabilising the interaction of p53 with DNA (R175, R282, G245) [37]. These seven mutations severely affect the ability of p53 to activate its transcriptional targets, whereas 24 of the other 27 rarer mutants retain transactivational capacity [34].

The human *TP53* knock-in (Hupki) mouse: an experimental model to study human *TP53* mutagenesis

The frequency and variety of *TP53* mutations in human cancer make it a useful target gene for experimental mutagenesis. A useful model for studying human *TP53* mutagenesis is a partial human *TP53* knock-in (Hupki) mouse (The Jackson Laboratory Repository designation: 129Trp53$^{tm/Holl}$) containing exons 4–9 of human *TP53* in place of the corresponding mouse exons (Figure 1) [38]. This mouse expresses a chimeric p53 protein that functions normally, whereas the p53 product of a full-length human *TP53* mouse model was functionally deficient [39]. Hupki mice homozygous for the knock-in allele do not develop spontaneous tumours at an early age, in contrast to *Tp53*-null mice [38]. Additionally, Hupki mice did not differ in tumour response from their counterparts with murine *Tp53* in a *N*-nitrosodiethylnitrosamine-induced hepatocarcinogenesis model [40]. Further, gene expression profiles from the spleens of untreated and γ-irradiated Hupki mice were highly concordant to those of WT mice, and key p53-target genes such as *Bax*, *Mdm2*,

and *Cyclin G* were induced by γ-irradiation. This indicates that the DNA damage response and transcriptional activities of p53, at least in the spleen, are similar in both mouse strains [38].

The Hupki mouse is useful for both *in-vitro* and *in-vivo* studies of *TP53* mutations induced by carcinogens. The nucleotide sequence of the mouse *Tp53* DBD differs by 15% from the human sequence, and this difference may greatly impact experimentally-induced mutation spectra [41]. Thus, mice, and cells derived from them, containing the human *TP53* DBD sequence can be used to test hypotheses on the origin of *TP53* mutations found in human tumours [38,42].

For an *in-vitro* assay, embryo fibroblasts from the Hupki mouse can be examined for the generation and selection of *TP53* mutations. The challenge in creating a mammalian cell mutation assay using *TP53* as a target gene is to identify a strategy for selecting mutated cells. Commonly used *in vitro* mutation assays that utilise either non-mammalian genes (*e.g. lacI, lacZ*) [43] or human genes with no known role in cancer (*e.g HPRT*) [44], generally involve manipulating growth conditions to favour the mutated cells. In order to select for *TP53*-mutated cells Hollstein and coworkers [38,45] exploited the fact that cultured mouse embryo fibroblasts (MEFs), in contrast to human fibroblasts, can be immortalised by mutation of *Tp53* alone.

MEFs undergo p53-dependent senescence after approximately 10 population doublings when cultured under standard conditions (20% atmospheric oxygen). This appears to occur in response to accumulated oxidative damage, as MEFs grown at physiological oxygen tension (3% oxygen) do not senesce (Figure 2) [46]. However, mouse fibroblasts which develop mutations in certain genes, such as *Tp53*, can bypass senescence and become immortalised [47,48]. The immortalisation of human cells is more complex. Cultured human cells proliferate for 50 or more population doublings at 20% oxygen before entering replicative senescence, which is regulated by both the p53 and $p16^{INK4a}$/pRB pathways, and they do not undergo immortalisation spontaneously [49,50]. If replicative senescence is bypassed by mutation or expression of viral oncogenes, human cells will only divide for further 10-20 population doublings before entering a second process termed ‘crisis’ [50]. Replicative senescence and ‘crisis’ of human cells is due to shortening of telomeres. Human cells, unlike mouse cells, do not express telomerase, thus immortalisation requires reconstitution or upregulation of telomerase activity, in addition to alterations in the p53 and $p16^{INK4a}$/pRB pathways [51,52]. Therefore, unlike mouse fibroblasts, human cells cannot be immortalised in culture simply by disruption of *TP53*.

In order to study *TP53* mutagenesis, Hupki embryo fibroblast (HUF) cultures are treated with a mutagen to induce *TP53* mutations (Figure 2). The treated cultures, along with untreated control cultures, are then serially passaged in 20% oxygen. Cells containing mutations (*e.g.* in *TP53*) that allow bypass of p53-dependent senescence become established into immortalised

cultures, while the majority of cells undergo irreversible growth arrest and are selected against. A detailed protocol for the HUF immortalisation assay has been published [42], and when these guidelines are followed each culture of 0.4–2 × 10^5 primary HUFs (untreated or mutagen-treated) will result in an immortal cell line. Untreated cultures are thought to undergo spontaneous immortalisation due to mutations induced by the cell culture conditions (*e.g.* DNA damage by ROS resulting from growth at 20% oxygen). DNA from the immortal HUF clones can then be sequenced to identify *TP53* mutations. The mutations identified in HUF clones derived from mutagen exposure can then be compared with the profile of mutations found in tumours of individuals who were exposed to the agent of interest. Most HUF mutants identified to date are classified as "non-functional" according to a yeast-based functional assay, which is in accordance with the majority of human tumour mutations (see Supporting Table S1) [34,53]. Additionally, HUF mutant clones can be directly evaluated for the impact of each mutation on the ability of p53 to transactivate target genes (*i.e. Cdkn1a, Puma, Noxa*). Indeed, it was recently shown that a set of *TP53* mutant HUF cell lines lost their ability to induce p53 target genes, while HUF clones with WT *TP53* generally retained transactivational activity [53]..

Investigating human cancer aetiology using the HUF immortalisation assay

Thus far four environmental carcinogens have been examined using the HUF immortalisation assay: (*i*) UV light, (*ii*) benzo[*a*]pyrene (B[*a*]P), (*iii*) 3-nitrobenzanthrone (3-NBA) and (*iv*) aristolochic acid I (AAI) (Figure 3) [54-59]. In each case a unique *TP53* mutation pattern was generated in the HUF immortalisation assay that differed from that found in control HUFs that had undergone spontaneous immortalisation.

UV-induced human skin cancer

The major aetiological agent contributing to non-melanoma skin cancer is sunlight, which includes UV frequencies [20,21]. *TP53* is frequently mutated in these tumours, and C to T or CC to TT transitions at dipyrimidine sites have been observed as signature mutations after UV-irradiation. Hotspot mutations were located at codons 151/152, 245, 248, 278 and 286 in *TP53* [60]. Two major types of DNA photoproducts, cyclobutane pyrimidine dimers (CPDs) and (6-4) pyrimidine-pyrimidone photoproducts [(6-4)PPs] (Figure 4), have been mapped in *TP53* in UV-irradiated human cells at the DNA sequence level using ligation-mediated polymerase chain reaction (LM-PCR). UV-induced DNA adducts were found most frequently at codons 151, 278 and 286 [60]. When HUFs were exposed to UV prior to selecting for immortalisation, five out of 20 HUF cell lines generated contained *TP53* mutations; all five carried base changes at dipyrimidine sites of

TP53 (a total of eight *TP53* mutations were detected) (Figure 3) [54]. The major mutation type induced was a C to T transition, the hallmark mutation in UV-induced mutagenesis. Interestingly, one UV-derived HUF harboured 3 single-base substitutions at codons 248, 249 and 250, one of which (248) is a hotspot location in human skin cancer [54].

Tobacco smoke-associated lung cancer

Tobacco smoking causes lung cancer and tobacco smoke contains many thousands of chemicals, including carcinogenic PAHs such as B[*a*]P [61]. B[*a*]P is metabolically activated by cytochrome P450 (CYP) enzymes (*e.g.* CYP1A1, CYP1B1) and epoxide hydrolase to the ultimately reactive metabolite B[*a*]P-7,8-diol-9,10-epoxide (BPDE) [62] that reacts primarily at the N^2 position of guanine in DNA (dG-N^2-BPDE) (Figure 4). Using the HUF immortalisation assay 28 HUF cell lines were derived from B[*a*]P treatment carrying a total of 37 *TP53* mutations [55,57; M. Hollstein, personal communication]. The predominant mutation type was G to T transversion accounting for 49% of the total, followed by G to C (22%) and G to A (19%) mutations (Figure 5A). Codons 157 and 273 account for ten of the mutations (five each) (Figure 5A).

The mutation pattern observed in human lung cancer from smokers is dominated by the presence of G to T transversions (30%), followed by G to A transitions (26%), and the distribution of mutations along *TP53* is characterised by several hotspots, in particular at codons 157, 158, 175, 245, 248 and 273 (Figure 5B). At several *TP53* mutational hotspots that are common to all cancers, such as codons 248 and 273, a large fraction of mutations in lung cancer are G to T events but are almost exclusively G to A transitions in non-tobacco-related cancers [22]. Whereas G to A mutations can arise through deamination of methylated cytosines, G to T transversions can be a consequence of misreplication of bases covalently modified by bulky carcinogens, such as B[*a*]P and other PAHs. Using LM-PCR selective DNA adduct formation was observed at guanine positions in codons 157, 248 and 273 in *TP53* of normal human bronchial epithelial cells treated with BPDE [63]. Subsequently, mapping of other PAH-derived DNA lesions yielded mostly similar results [64], suggesting that the overall spectrum of *TP53* mutations in lung cancer of smokers is determined by exposure to multiple PAHs, possibly having additive or multiplicative effects. Interestingly, the G to T transversions observed in codons 157, 248 and 273 are at sites containing methylated CpG dinucleotides (all CpG sites in the DBD of *TP53* are completely methylated) [22]. It has been proposed that methylation at CpG sites may increase the potential for planar carcinogen compounds to intercalate prior to covalent binding, although the precise mechanism still needs to be determined. Furthermore, the majority of G to T transversions occur on the non-transcribed DNA strand, particularly at hotspot codons 157, 158 and 273, which may be linked to the fact that B[*a*]P-

derived DNA adducts are removed less efficiently from the non-transcribed strand than from the transcribed strand of this gene [23,65]. As already described, in cell lines from B[*a*]P-treated HUFs, codons 157 and 273 are also recurrent sites of mutation (Figure 5A) with a significant proportion of these mutations being G to T [57]. Consequently, the data collected in the HUF immortalisation assay are consistent with the hypothesis that B[*a*]P has a direct role in causing smokers' lung tumour *TP53* mutations.

3-Nitrobenzanthrone, a potential human lung cancer hazard in diesel exhaust and urban air pollution

Epidemiological studies suggest that air pollution may increase lung cancer risk [66]. Nitro-PAHs are present on the surface of ambient air particulate matter and diesel exhaust particles [67] and their detection in lungs of non-smokers with lung cancer has led to considerable interest in assessing their potential risk to humans [68]. The aromatic nitroketone 3-NBA (Figure 4) is one of the most potent mutagens and potential human carcinogens identified in diesel exhaust and ambient air pollution [69-71]. Indeed, 3-NBA induces squamous cell carcinoma in rat lung after intratracheal administration [70]. 3-NBA forms DNA adducts after metabolic activation through reduction of the nitro group, which is primarily catalysed by NAD(P)H:quinone oxidoreductase (NQO1) [72,73]. It can be further activated by *N*-acetyltransferase and sulfotransferases [72,74]. The predominant DNA adducts detected *in vivo* in rodents after treatment with 3-NBA are 2-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone (dG-N^2-3-ABA) and *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-*N*-3-ABA) [75,76] (Figure 4), and these are most probably responsible for the G to T transversion mutations induced by 3-NBA in transgenic MutaMouse [77].

Using the HUF immortalisation assay 19 cell lines carrying a total of 29 *TP53* mutations were derived from 3-NBA treatment [59]. The major mutation type induced by 3-NBA was G to T transversion (38%), followed by A to G (24%) and G to C (17%) mutations (Figure 5C). Although G to T transversions were also the predominant mutations found in B[*a*]P-treated HUFs, the mutation spectra for 3-NBA and B[*a*]P were significantly different [59], indicating that each carcinogen likely has a characteristic mutation signature. A large number of 3-NBA-induced mutations were found at adenine residues (total 44%), which is in line with the fact that 3-NBA also binds covalently at adenine (*e.g.* 2-(2'-deoxyadenosin-N^6-yl)-3-aminobenzanthrone) [75], although nothing is yet known about the mutagenic potential of those adducts using a site-specific mutagenesis assay.

In lung tumours of non-smokers G to A transitions (40%) and G to T transversions (17%) are the prominent types of mutations induced (Figure 5D). G to T transversions have also been

detected at high frequency in the lungs of *gpt*-delta transgenic mice following inhalation of diesel exhaust [78]. Furthermore, in the same model the mutations induced by 1,6-dinitropyrene, another nitro-PAH present in diesel exhaust, were mainly G to A transitions and G to T transversions [79]. Therefore, it is tempting to speculate that nitro-PAHs, including 3-NBA, may contribute to the induction of G to T mutations in lung tumours of non-smokers.

Aristolochic acid-exposed human urothelial cancer

The herbal drug AA, which comes from the genus *Aristolochia*, has been associated with the development of a novel human nephropathy, known as aristolochic acid nephropathy (AAN), and its associated urothelial cancer [80,81]. AAI (Figure 4) is the major component of the plant extracts. AAN was first reported in Belgian women who had consumed Chinese herbs as part of a weight-loss regimen in 1991 and was traced to the ingestion of *Aristolochia fangchi* inadvertently included in the slimming pills [81]. Within a few years of taking the pills, AAN patients had developed a high risk of upper tract urothelial carcinoma (about 50%) [82] and, subsequently, bladder urothelial carcinoma [83]. Using the highly sensitive ^{32}P-postlabelling assay, exposure to AA was demonstrated by the identification of specific AA-DNA adducts in urothelial tissue of AAN patients [82,84,85]. Further, chronic exposure to *Aristolochia clematitis* has been linked to BEN and its associated urothelial cancer [26,27]. This nephropathy is endemic in certain rural areas of Serbia, Bosnia, Croatia, Bulgaria and Romania. BEN is clinically and morphologically very similar to AAN; indeed, AA-specific DNA adducts have been detected in BEN patients and in individuals with end-stage renal disease living in areas endemic for BEN [27,86], suggesting that dietary exposure to AA is a risk factor for the development of the disease.

The major activation pathway of AA is reduction of the nitro group (Figure 4). Cytosolic NQO1 has been shown to be the most efficient enzyme, although CYP1A1, CYP1A2 and prostaglandin H synthase (cyclooxygenase, COX) are also able to metabolically activate AA [87]. The most abundant DNA adduct detected in AAN and BEN patients is 7-(deoxyadenosin-N^6-aristolactam I (dA-AAI), and A to T tranversion mutations in *TP53* are found in the urothelial tumours associated with both pathologies (see below) [27,88]. *In-vitro* experiments using terminal tranferase-dependent PCR (TD-PCR) analysis have revealed that AA preferentially binds to purine bases within *TP53* [89].

To date 32 immortalised HUF cell lines have been derived from AAI treatment carrying a total of 37 *TP53* mutations [54,56,58]. The AAI-induced *TP53* mutation pattern is dominated by A to T transversions (57%) (Figure 6A). One of the experimentally-induced A to T mutations (at codon 139) matches the *TP53* mutation reported in a urothelial carcinoma of an AAN patient in the

UK [88]. In urothelial tumours of BEN patients from Croatia (*n*=11), mutations at A:T pairs accounted for 89% (17/19) of all mutations, with the majority of these (15/17) being A to T transversions, representing 78% of all base substitutions detected in *TP53* (Figure 6B) [27]. In contrast, A to T transversions account for only around 5% of all the *TP53* mutations in non AA-associated human urothelial tumours (Figure 6C). Strikingly, eight of the A to T mutations in AAI-treated HUFs (at codons 131 [2×], 209 [3×], 280, 286 and 291) are uncommon in the IARC TP53 database but are identical to mutations found in urothelial tumours from BEN patients (at codons 131, 209, 280 [3×], 286 and 291 [2×]) [27,58]. Given that the *TP53* mutations in tumours of BEN patients correlate remarkably well with AAI-HUF experimental mutations, yet are of a type rare in other human urothelial tumours, this strongly suggests that AA plays a causative role in the aetiology of BEN-associated tumourigenesis [58]. IARC recently classified AA as a human (Group 1) carcinogen (having previously classified it in Group 2A [probable human carcinogen] in 2000) [90]. This example illustrates how mechanistic data, including that obtained by the HUF immortalisation assay, can help to identify human carcinogenic hazards.

Current limitations and possible future modifications for the HUF immortalisation assay

Despite the utility of the current HUF immortalisation assay, it has several limitations that could be addressed by future developments. Firstly, the assay does not specifically select for *TP53*-mutated cells, but rather for bypass of senescence induced by hyperoxic cell culture conditions. Modification of genes other than *TP53* can allow MEFs to avoid the p53-controlled arrest induced by oxidative stress and to undergo immortalisation. For example, besides *TP53* mutation, the most commonly found genetic alteration in immortalised MEFs is loss of the $p19^{Arf}$ locus [48]. However, a recent study showed that loss of $p19^{Arf}$ occurs in only 5% of spontaneously immortalised HUF cell lines compared with 17% of immortalised MEFs with the nascent *Tp53* gene [53]. A number of other cancer-associated genes have been shown to regulate MEF senescence, including *Mdm2*, *Cdk4*, *Tbx2*, *Bcl6*, *GSK3β*, and several others [91-96]. The proportion of immortalised HUF clones with mutated *TP53* is up to 20% in spontaneously immortalised cultures and up to 40% in treated cultures depending on the mutagen [53,57,59]. Thus, the majority of immortalised HUF cell lines do not contain *TP53* mutations and the effort expended culturing these clones is fruitless. If possible, a new or additional selection procedure specific to the activity of only p53 would be a great improvement to the assay and further work will be required to develop such a procedure.

An additional aspect of the assay to consider is the paradox presented by the growth of HUFs in 20% oxygen. On one hand, this level of oxygen is necessary to serve as the selective pressure for the growth of HUFs with mutant p53 in the immortalisation assay; conversely, growth

under atmospheric oxygen leads to oxidative damage and mutations [46]. Using a *lacZ* reporter gene, it has been shown that MEFs grown in 20% oxygen accumulate point mutations as they become immortalised. After 17 population doublings, the majority of mutations are G to T transversions, a signature mutation of oxidatively damaged DNA [97]. MEFs grown in 3% oxygen, on the other hand, do not accumulate such mutations over at least 20 population doublings. Thus, HUFs are likely to acquire ROS-induced DNA lesions throughout culturing and the immortalisation process at 20% oxygen, both before and after treatment with a mutagen. These mutations could be within *TP53* itself, or in one of the other genes capable of regulating senescence, and may contribute to the background frequency (*i.e.* not induced by mutagen treatment) of mutation and immortalisation.

In order to clarify the origin of mutations in the assay, it is necessary to compare the *TP53* mutation pattern of spontaneously immortalised HUFs (the untreated controls) to that of mutagen-treated HUFs. Interestingly, previous studies have shown that the most common type of *TP53* mutation in the spontaneously immortalised HUFs is G to C transversion (Figure 3), whereas G to T transversion, the type most commonly associated with oxidatively-damaged DNA, is infrequent [53]. Although ROS-damaged DNA can also result in G to C transversions [98], it is as yet unclear why G to T transversions are not also common in *TP53* in HUFs spontaneously immortalised by growth in 20% oxygen. Regardless, one would hypothesise that limiting the exposure of HUFs to hyperoxic conditions would be likely to reduce the level of background mutations, whatever type they may be, if the assumption that they are indeed caused by ROS is correct. Cells could be maintained under 3% oxygen both before and during mutagen treatment, and then transferred to 20% oxygen to select for senescence bypass. Furthermore, if an alternative to incubation in 20% oxygen for selecting *TP53*-mutated cells were to be developed (see above), the entire assay could potentially be performed solely under 3% oxygen.

Taking cues from other mutagenesis systems, such as the Salmonella Ames assay, further modifications to the HUF immortalisation assay could include (*i*) enhancement of xenobiotic metabolism to increase the range of chemical carcinogens which can be tested and (*ii*) modification of DNA repair processes to increase the mutation frequency. Xenobiotic metabolism, which is responsible for activating pro-carcinogens into DNA-reactive intermediates, can differ significantly between species and cell types [99]. HUFs have been shown to express many key metabolic enzymes, such as CYPs, but they have not been fully characterised and may be metabolically incompetent for some types of chemical pro-carcinogens [54]. For such compounds it could be advantageous to co-incubate cells with hepatic S9 fractions or isolated microsomes, which are enriched in many xenobiotic metabolism enzymes (*e.g.* CYPs) [100]. Alternatively, Hupki mice

could be created, by genetic engineering or crossbreeding, that express or over-express desired enzymes. For example, in mice expressing human CYP1A2 (knocked-in to replace the mouse gene) the food mutagen 2-amino-1-methyl-6-phenylimidazo[*4,5-b*]pyridine (PhIP), is preferentially hydroxylated at the N^2-position (*i.e.* activation), whereas in WT mice expressing innate Cyp1a2, 4'-hydroxylation (*i.e.* detoxification) is the predominant pathway [101,102]. Thus, expression of human CYPs in Hupki mice could be an important refinement for assessing the mutagenic potential of selected compounds like PhIP that are not well activated by the nascent WT mouse enzymes.

Finally, the assay could be made more sensitive by interfering with the ability of HUFs to repair certain types of DNA damage. This could be achieved by genetic engineering or by crossbreeding to generate Hupki mice deficient in nucleotide excision repair (NER) or base excision repair (BER). For example, mice deficient in the Xeroderma pigmentosum group A (*Xpa*) gene are defective in NER and highly susceptible to environmental carcinogens [103]. Many bulky DNA adducts (*e.g.* those formed by B[*a*]P, 3-NBA or AAI) are removed via the NER pathway and *Xpa*-null mice exhibit increased mutation frequency in *lacZ* after treatment with adduct-forming compounds [104,105]. We have recently crossed *Xpa*-deficient mice with the Hupki mice in order to study the role of Xpa function and NER on the induction of *TP53* mutations in HUFs. It is anticipated that DNA-repair deficient HUFs should be more susceptible to environmental mutagens, providing a more sensitive assay to screen for *TP53* mutations. However, it may also be important to consider that mouse cells differ from human cells in some aspects of DNA repair, and this may affect the *TP53* mutation spectrum observed in HUFs, perhaps depending on the type of mutagen. For example, mouse cells are deficient in the global genomic repair of UV-induced CPDs, which has been attributed to a lack of p48 protein expression [106]. Due to this deficiency, UV-induced skin tumours in hairless mice contain *Tp53* mutations predominantly on the non-transcribed DNA strand, whereas in humans there is no strand bias [107].

In-vivo Hupki studies

In addition to *in-vitro* studies, the Hupki mouse can be used to study *in-vivo TP53* mutagenesis in carcinogen-induced tumours [108,109]. The utility of such studies may be limited, however, because, with the exception of skin carcinomas, the majority of chemically-induced or spontaneous tumours in mice do not necessarily contain *Tp53* mutations [110]. This observation is perplexing, considering the fact that both *Tp53*-null mice and mice genetically engineered to express mutant *Tp53* readily develop tumours. The reason(s) for the discrepancy is still unclear, but several hypotheses have been proposed [110,111]. For instance, whether or not nascent mouse *Tp53* is found mutated in tumours appears to depend at least in part on the treatment protocol and target

organ. It may also depend on the genetic background of a given mouse strain, or on fundamental differences in the signalling pathways and/or regulation of growth control genes between mice and humans [111].

To date, only UVB irradiation of Hupki mice has resulted in tumours containing *TP53* mutations [108]. When Hupki mice were irradiated with a single acute dose of UVB, DNA lesion footprinting showed an accumulation of UV photoproducts in their epidermal DNA at the same locations within *TP53* as were found in human cells [108]. Furthermore, after chronic UVB exposure for several weeks Hupki skin epidermal cells harboured C to T and CC to TT transitions at two mutation hotspots (codons 247/248 and 278/279) identified in human skin cancer [108]. In contrast, no *Tp53* mutations were found at sequences equivalent to human codons 247/248 in UVB-induced skin tumours of WT mice [41], indicating that Hupki mice can reproduce *TP53* hotspot alterations typically found in sunlight-exposed human skin of healthy individuals and of UV-associated human tumours. In the only other *in-vivo* study performed thus far to examine *TP53* mutagenesis, no *TP53* mutations were found in aflatoxin B_1-induced liver tumours of Hupki mice, although these mice showed enhanced susceptibility to carcinogenesis relative to WT mice [109].

Hupki mice can also be genetically modified to express common human cancer-associated *TP53* mutations in order to study their effect on tumourigenesis. By introducing the mutation into the humanised *TP53* allele rather than WT mouse *Tp53*, the impact of the mutation on the structure and function of human p53 may be more accurately reproduced. Song *et al.* [112] engineered Hupki mice to express two of the most common p53 cancer mutants, R248W and R273H, designated $TP53^{R248W}$ and $TP53^{R273H}$, respectively. $TP53^{R248W}$ mice developed tumours at a rate similar to *Tp53*-null mice (data for the tumourigenesis in $TP53^{R273H}$ mice was not presented). However, in addition to the thymomas and sarcomas formed in *Tp53*-null mice, $TP53^{R248W}$ mice also developed lymphomas and germ-cell tumours. The sarcomas in $TP53^{R248W}$ mice included haemangiosarcomas and rhabdomyosarcomas, which are rarely observed in *Tp53*-null mice. This difference in tumour spectrum suggests a gain-of-function activity for the R248W mutation. The authors went on to show that cells expressing the R248W or R273H mutants had enhanced genetic instability and an impaired DNA damage response. It would be interesting to see how other *TP53* mutations expressed in the Hupki mouse model affect p53 activity and tumour development.

Codon 72 polymorphic variants of the Hupki mouse

A common polymorphism occurs at codon 72 in *TP53*, resulting in either a proline (Pro72) or an arginine (Arg72). This occurs in the region of the gene encoding the polyproline domain, which is important for the apoptotic functions of p53 [113]. Interestingly, the polymorphism has

been suggested to influence the biology of p53 and selection of *TP53* mutations and, in turn, cancer risk and response to therapy [114]. The original Hupki mouse contains Arg72, but Pro72 and Pro/Arg72 variant strains have been generated subsequently [57]. HUFs from these mice can be used to study the mutability of the polymorphic alleles and their impact on normal and mutant p53 function.

There is some evidence that the Arg72 variant of p53 may be better at inducing apoptosis. Using cancer cell lines engineered to express either the Pro72 or Arg72 variant of p53 (referred to here as p53-Pro72 and p53-Arg72, respectively), p53-Arg72 was at least five times better at inducing apoptosis than p53-Pro72 [115]. The difference was associated with enhanced localisation of p53-Arg72 to the mitochondria. A study by Bonafe *et al.* [116] showed that normal cells expressing the p53-Arg72 variant have increased apoptosis in response to oxidative stress compared with cells expressing p53-Pro72, but this was only significant in cells from older patients.

If p53-Arg72 has an enhanced ability to induce apoptosis, it might be better able to protect individuals from cancer by eliminating damaged cells, but mutations on this allele may then be preferentially selected for. Some studies have indeed reported more frequent mutation of the Arg72 allele in cancer [117,118]. Additionally, it was found that mutant p53-Arg72 binds more strongly to and inhibits p73 than does mutant p53-Pro72, leading to gain-of-function activity and a selective growth advantage [117]. However, there are other studies showing that the Pro72 allele is more frequently mutated in human tumours [119-121].

HUFs containing the three allelic configurations of the codon 72 polymorphism (Pro/Pro, Arg/Arg, Pro/Arg) can be used to study various hypotheses regarding the effect of this polymorphism in the selection of mutations on the variant alleles, as well as in p53 apoptotic function. For example, heterozygous Hupki$^{Pro/Arg72}$ HUFs could be used to determine whether mutations on one variant allele are more frequently selected for in the immortalisation assay. Interestingly, a study on AA-induced mutations by Reinbold *et al.* [57] showed a trend for more frequent mutation of the proline allele, with corresponding loss of the arginine allele in immortalised Hupki$^{Pro/Arg72}$ fibroblasts. As another possibility, HUF cell lines generated in the mutagenesis assay which contain *TP53* mutations *in cis* with either the Pro72 or Arg72 polymorphism could be used to assess how the polymorphism influences the activities of mutant p53.

Concluding remarks

With the mutagenic agents examined thus far, all of which are strongly genotoxic, it is apparent that the HUF immortalisation assay has sufficient specificity to make it applicable to a

wide range of other agents that putatively play a role in the aetiology of human cancer. Comparison of the *TP53* mutation spectra generated by the *in-vitro* assay with the spectra of mutations in human tumours may test such hypotheses. Nevertheless, it is apparent from the studies conducted to date that there is considerable scope for improving both the sensitivity of the assay (the number of *TP53* mutants generated in each assay are relatively few) and its selectivity (only a proportion of the immortalised HUF clones actually contain mutated *TP53*). Further development of the assay to address these shortcomings offers the possibility of wider application of the assay to investigate some of the many outstanding uncertainties about cancer aetiology, in ways that are closely related mechanistically to the molecular pathology of the disease.

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Legend to Figures:

Figure 1:

Generation of the human *TP53* knock-in (Hupki) mouse (adapted from reference [38]). A targeting vector was created containing (*i*) exons 2–3 of mouse *Tp53* sequence, (*ii*) a loxP-flanked neomycin (Neo) resistance cassette, (*iii*) exons 4–9 (and flanking introns) of human *TP53*, and (*iv*) exon 10 of mouse *Tp53*. The targeting vector was electroporated into embryonic stem (ES) cells which were subsequently selected for neomycin resistance and screened for recombination at exons 2–3 and exon 10 by PCR and Southern blotting. Correctly targeted ES clones were transfected with a Cre-expressing vector to delete the loxP-flanked neomycin cassette, yielding the final human *TP53* knock-in (*Hupki*) allele. ES clones with the Hupki allele were injected into C57BL/6 blastocysts to generate chimeric mice, which were then backcrossed to 129/Sv mice.

Figure 2:

Experimental scheme of the HUF immortalisation assay. Primary fibroblasts are isolated from Hupki mouse embryos (passage 0) and seeded on multi-well plates (*i.e.* 40,000 cells/well on 24-well plates or 200,000 cells/well on 6-well plates). Cells are treated with a test agent (*e.g.* environmental mutagen) at passage 0 or 1 (control cultures are treated with solvent). Cells are then serially passaged at 20% oxygen until the majority of each culture undergoes senescent crisis due to oxidative stress (between passage 4 to 8). Cells that have not senesced will continue to grow and will emerge as immortalised, clonal cell lines after at least 10 passages. These cultures often contain missense mutations in *TP53*. Isolated DNA is sequenced for mutations in *TP53* to assess the effect of the mutagen on the pattern and spectrum of mutations. Inserts: Morphology of HUFs at different stages of the HUF immortalisation assay. Photomicrographs of cells growing in adherent monolayers were taken at 10× magnification. Primary HUFs become enlarged and flattened during senescence. Cells which bypass senescence grow into immortalised clonal populations of homogenous appearance; different sizes and morphologies of immortalised clones are observed (data not shown).

Figure 3:

Comparison of the types of *TP53* base substitutions found in immortalised HUF cell lines treated with UV light [54], B[*a*]P [55,57], 3-NBA [59] or AAI [54,56,58]. Also shown is the mutation pattern in spontaneous immortalised HUFs (controls) [53].

Figure 4:

Environmental carcinogens that have been investigated in the HUF immortalisation assay, their major sites of DNA modification, and major type of induced mutation. DNA adducts have been structurally identified as: (6-4)PP, (6-4) pyrimidine-pyrimidone photoproduct; CPD, cyclobutane pyrimidine dimer; dG-N^2-BPDE, 10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; dG-N^2-3-ABA, 2-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone; dG-C8-*N*-3-ABA, *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone; dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I.

Figure 5:

Mutation pattern and spectra of *TP53* mutations in immortalised HUF cell lines treated with B[*a*]P (A) [55,57; Monica Hollstein, personal communication] or 3-NBA (C) [59]. Also shown is the mutation pattern and spectra of *TP53* mutations in spontaneously immortalised HUFs (controls) (E) [53]. *TP53* mutation pattern and spectra in lung cancer of smokers (B) or nonsmokers (D). Mutation data from human tumours were obtained from the IARC TP53 mutation database (www.p53.iarc.fr; R13 version). Entries with confounding exposure to asbestos, mustard gas or radon were excluded. Note that in the mutations spectrum only single base substitutions in codons are shown here; single base substitution detected for instance at splice sites are not depicted.

Figure 6:

(A) Mutation pattern and spectrum of *TP53* mutations in immortalised HUF cell lines treated with AAI [54,56,58]. (B) *TP53* mutation pattern and spectra in BEN-associated urothelial cancer [27]. Codons containing A to T transverion mutations are indicated by asterisks (*). (C) *TP53* mutation pattern and spectra in urothelial cancer not associated with AA exposure. Mutation data from human tumours were obtained from the IARC TP53 mutation database (www.p53.iarc.fr; R13 version). Organs included are kidney, bladder, renal pelvis, ureter and other urinary organs. Morphology inclusion criteria: carcinoma not otherwise specified, carcinoma *in situ* not otherwise specified, dysplasia not otherwise specified, papillary carcinoma not otherwise specified, papillary transitional cell carcinoma, transitional cell carcinoma not otherwise specified, transitional cell carcinoma *in situ*, squamous cell carcinoma not otherwise specified, and urothelial papilloma not otherwise specified. Note that in the mutation spectrum only single base substitutions in codons are shown here; single base substitution detected for instance at splice sites are not depicted.

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Figure 1

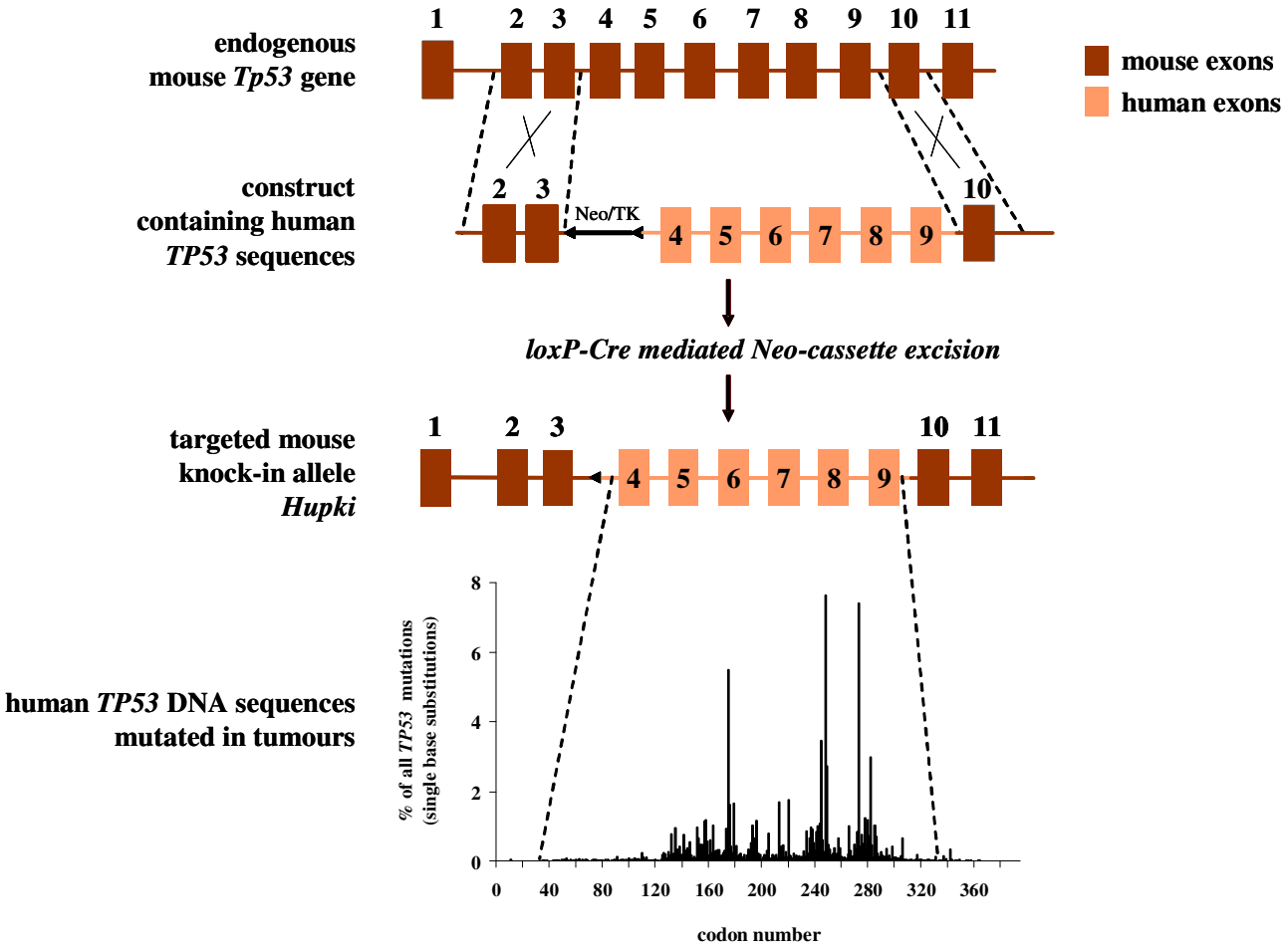

endogenous mouse *Tp53* gene
1 2 3 4 5 6 7 8 9 10 11
mouse exons
human exons
construct containing human *TP53* sequences
2 3 Neo/TK 4 5 6 7 8 9 10
loxP-Cre mediated Neo-cassette excision
targeted mouse knock-in allele *Hupki*
1 2 3 4 5 6 7 8 9 10 11
human *TP53* DNA sequences mutated in tumours
% of all *TP53* mutations (single base substitutions)
0 2 4 6 8
0 40 80 120 160 200 240 280 320 360
codon number

Figure 2

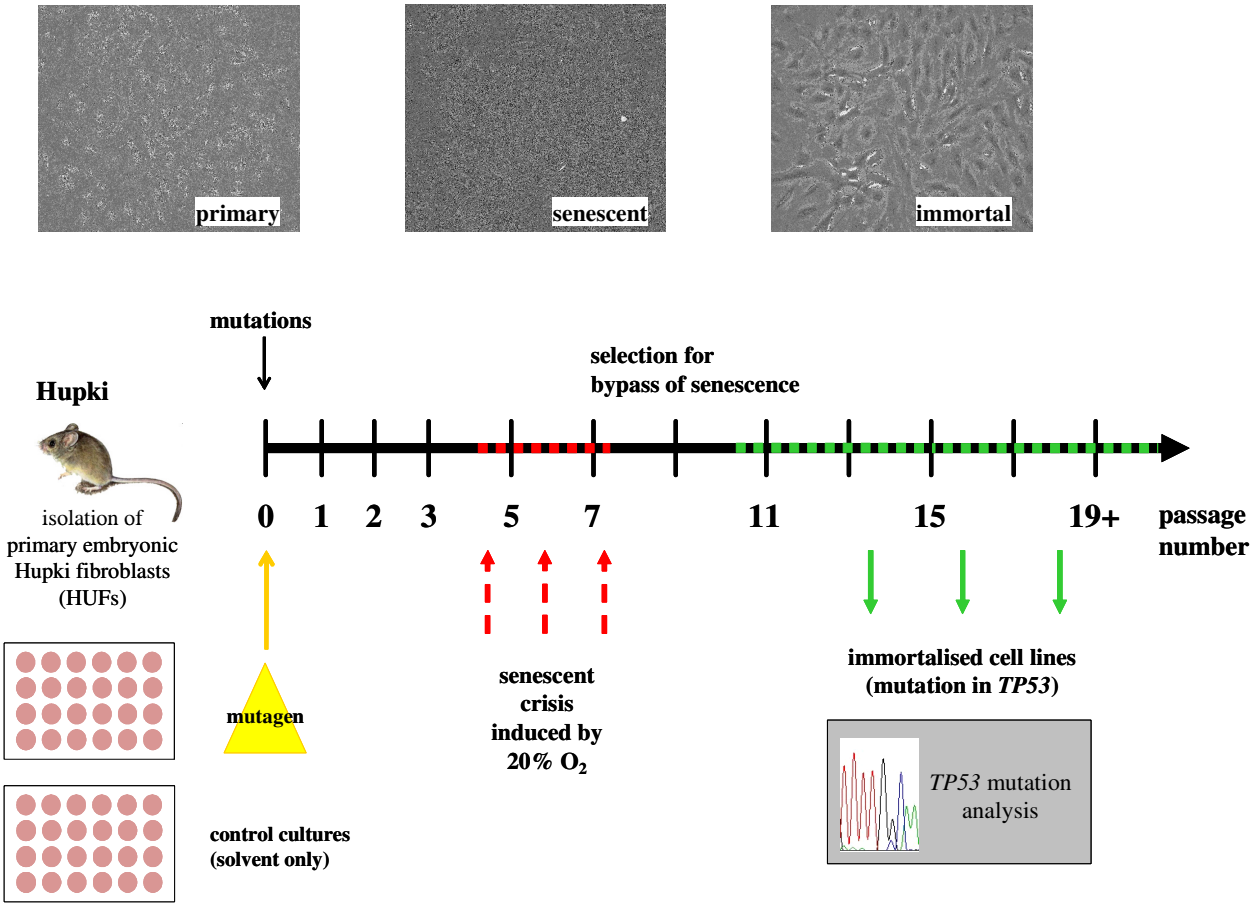

primary
senescent
immortal
mutations
selection for
bypass of senescence
Hupki
isolation of
primary embryonic
Hupki fibroblasts
(HUFs)
0 1 2 3 5 7 11 15 19+
passage
number
mutagen
control cultures
(solvent only)
senescent
crisis
induced by
20% O2
immortalised cell lines
(mutation in TP53)
TP53 mutation
analysis

Figure 3

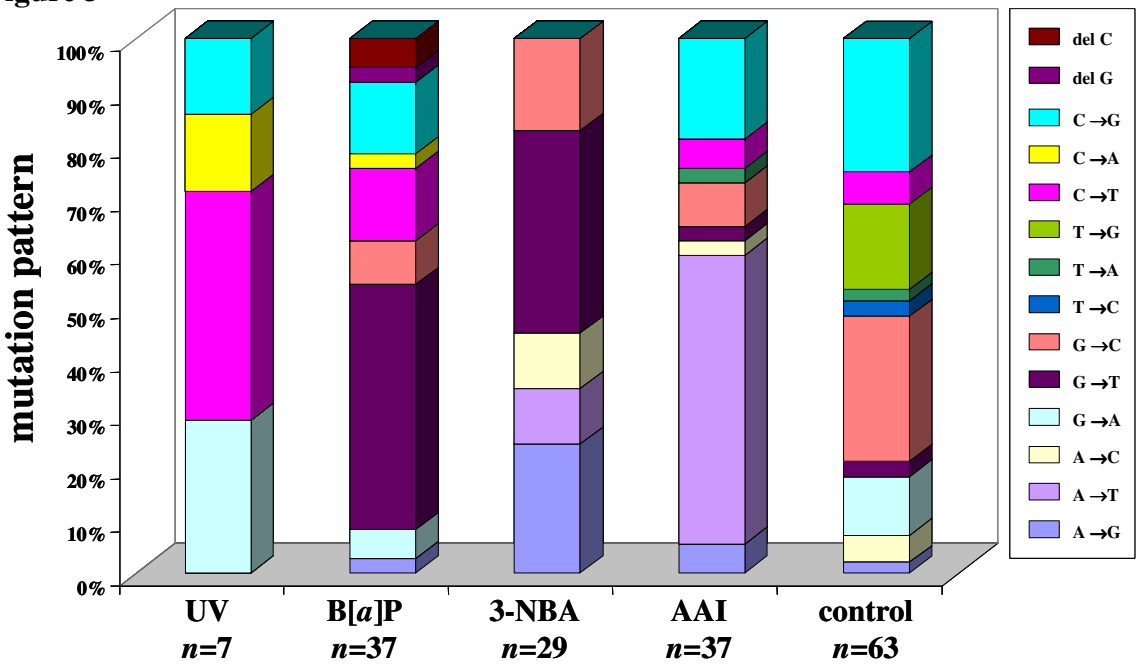

mutation pattern
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
UV
n=7
B[a]P
n=37
3-NBA
n=29
AAI
n=37
control
n=63
del C
del G
C →G
C →A
C →T
T →G
T →A
T →C
G →C
G →T
G →A
A →C
A →T
A →G

Figure 4

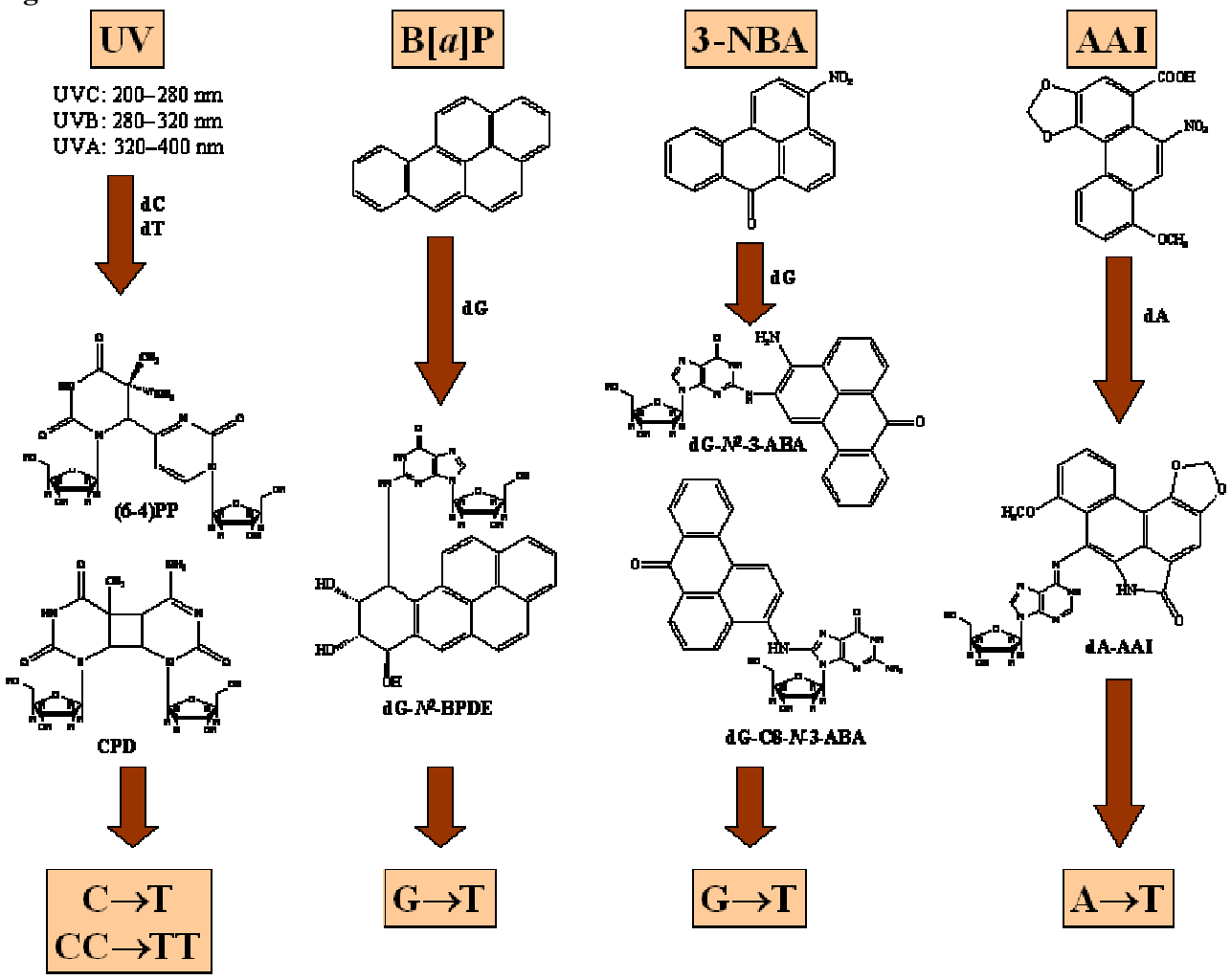

UV
UVC: 200–280 nm
UVB: 280–320 nm
UVA: 320–400 nm
dC
dT
(6-4)PP
CPD
C→T
CC→TT
B[a]P
dG
dG-N²-BPDE
G→T
3-NBA
dG
dG-N²-3-ABA
dG-C8-N-3-ABA
G→T
AAI
dA
dA-AAI
A→T

Figure 5

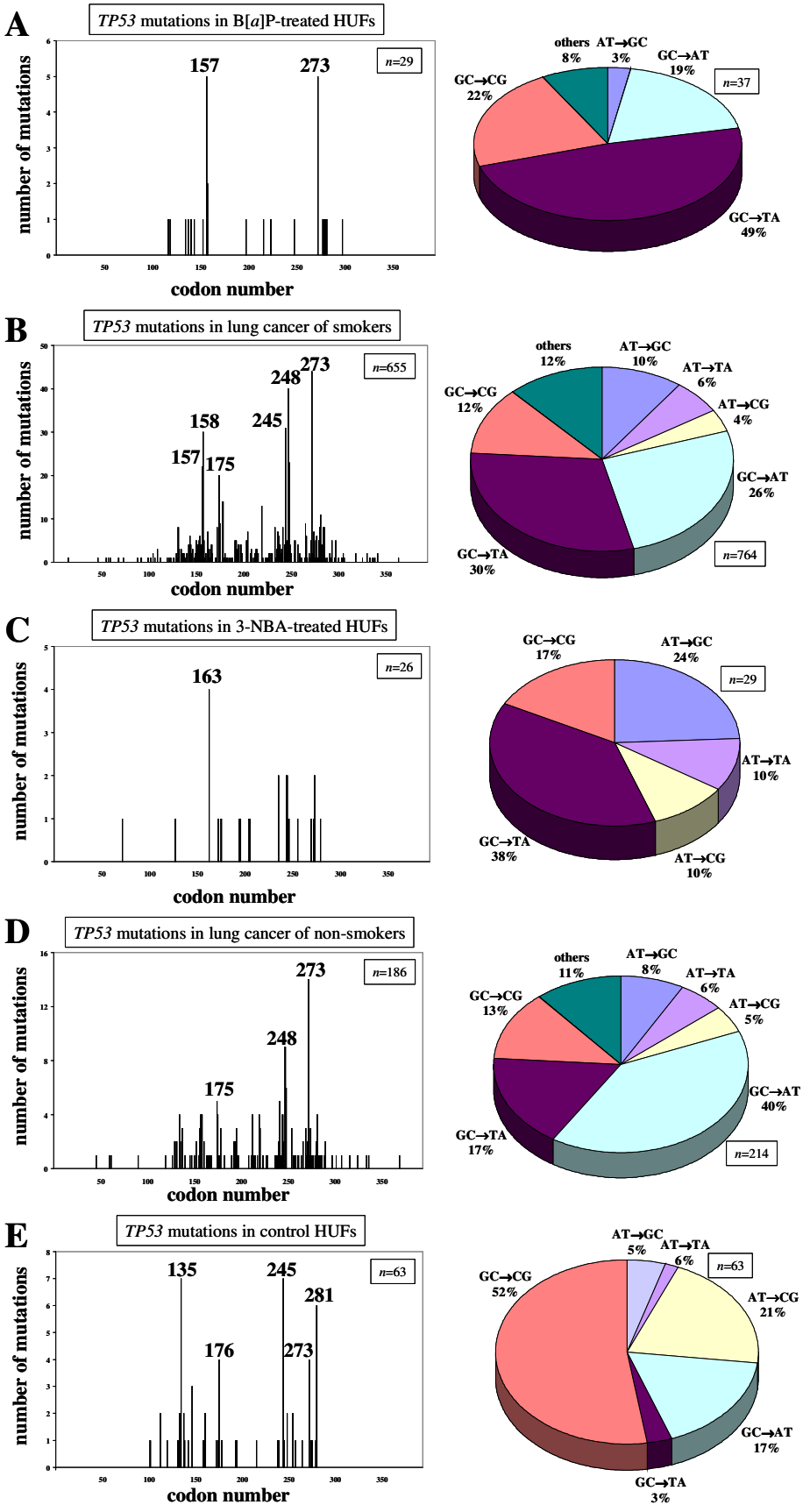
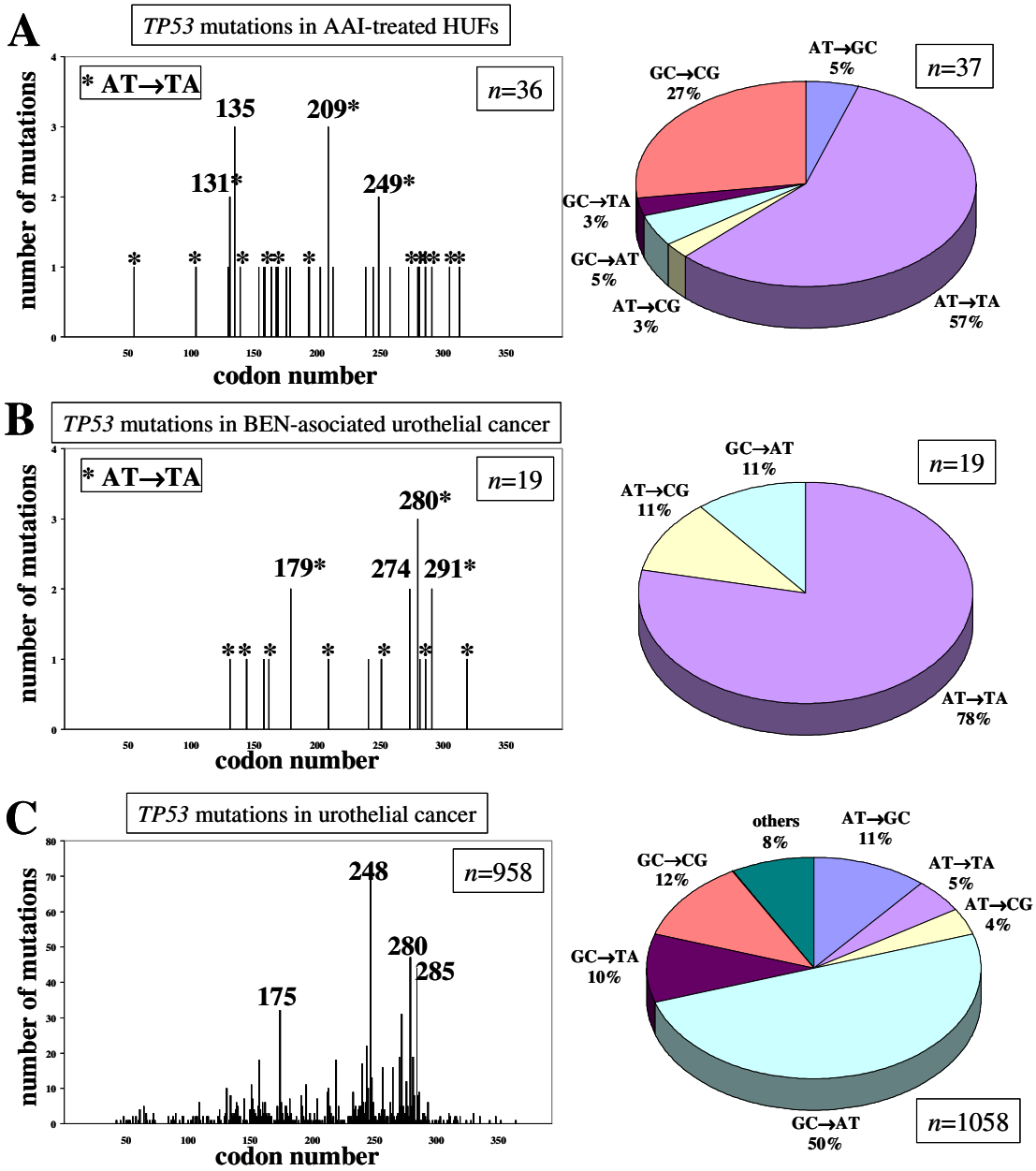

A
TP53 mutations in B[a]P-treated HUFs
number of mutations
codon number
157
273
n=29
others 8%
AT→GC 3%
GC→AT 19%
GC→CG 22%
GC→TA 49%
n=37
B
TP53 mutations in lung cancer of smokers
number of mutations
codon number
273
248
158
245
157
175
n=655
others 12%
AT→GC 10%
AT→TA 6%
AT→CG 4%
GC→CG 12%
GC→AT 26%
GC→TA 30%
n=764
C
TP53 mutations in 3-NBA-treated HUFs
number of mutations
codon number
163
n=26
GC→CG 17%
AT→GC 24%
n=29
AT→TA 10%
GC→TA 38%
AT→CG 10%
D
TP53 mutations in lung cancer of non-smokers
number of mutations
codon number
273
248
175
n=186
others 11%
AT→GC 8%
AT→TA 6%
GC→CG 13%
AT→CG 5%
GC→AT 40%
GC→TA 17%
n=214
E
TP53 mutations in control HUFs
number of mutations
codon number
135
245
281
176
273
n=63
AT→GC 5%
AT→TA 6%
n=63
GC→CG 52%
AT→CG 21%
GC→AT 17%
GC→TA 3%

Figure 6


A
TP53 mutations in AAI-treated HUFs
* AT→TA
n=36
135
209*
131*
249*
number of mutations
codon number
AT→GC 5%
GC→CG 27%
n=37
GC→TA 3%
GC→AT 5%
AT→CG 3%
AT→TA 57%
B
TP53 mutations in BEN-asociated urothelial cancer
* AT→TA
n=19
280*
179*
274
291*
number of mutations
codon number
GC→AT 11%
AT→CG 11%
n=19
AT→TA 78%
C
TP53 mutations in urothelial cancer
n=958
248
280
285
175
number of mutations
codon number
others 8%
AT→GC 11%
GC→CG 12%
AT→TA 5%
AT→CG 4%
GC→TA 10%
GC→AT 50%
n=1058